Environ. Sci. Technol. **2010,** *44,* 2454–2460

Gas Phase Measurements of Pyruvic Acid and Its Volatile Metabolites

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Received November 22, 2009. Revised manuscript received February 12, 2010. Accepted February 18, 2010.

Pyruvic acid, central to leaf carbon metabolism, is a precursor of many volatile organic compounds (VOCs) that impact air quality and climate. Although the pathways involved in the production of isoprenoids are well-known, those of several oxygenated VOCs remain uncertain. We present concentration and flux measurements of pyruvic acid and other VOCs within the tropical rainforest (TRF) biome at Biosphere 2. Pyruvic acid concentrations varied diurnally with midday maxima up to 15 ppbv, perhaps due to enhanced production rates and suppression of mitochondrial respiration in the light. Branch fluxes and ambient concentrations of pyruvic acid correlated with those of acetone, acetaldehyde, ethanol, acetic acid, isoprene, monoterpenes, and sesquiterpenes. While pyruvic acid is a known substrate for isoprenoid synthesis, this correlation suggests that the oxygenated VOCs may also derive from pyruvic acid, an idea supported by leaf feeding experiments with sodium pyruvate which resulted in large enhancements in emissions of both isoprenoids and oxygenated VOCs. While feeding with sodium pyruvate-2-^{13}C resulted in large emissions of both ^{13}C-labeled isoprenoids and oxygenated VOCs, feeding with sodium pyruvate-1-^{13}C resulted in only ^{13}C-labeled isoprenoids. This suggests that acetaldehyde, ethanol, and acetic acid are produced from pyruvic acid via the pyruvate dehydrogenase (PDH) bypass system (in which the 1-C carbon of pyruvic acid is lost as CO_2) and that acetone is also derived from the decarboxylation of pyruvic acid.

Introduction

Pyruvic acid lies at the heart of primary carbon and energy metabolism in plants, acting at the intersection of several key metabolic pathways (Figure 1). It is the end product of glycolysis and is also produced from pathway intermediates such as glyceraldehyde-3-phosphate from the Calvin cycle. It is converted to acetyl CoA by the action of pyruvate dehydrogenase (PDH) during aerobic respiration in mitochondria and fatty acid biosynthesis in plastids. Alternatively, it can be converted in the cytosol to acetaldehyde by pyruvate decarboxylase (PDC) and subsequently reduced to ethanol or converted to acetyl CoA via the PDH bypass. The PDH bypass involves oxidation of acetaldehyde by aldehyde dehydrogenase (ALDH), forming acetic acid which is then activated to acetyl CoA by acetyl CoA synthetase (ACS). ACS activity is considered to be mainly localized to plastids where it supplies acetyl CoA for fatty acid biosynthesis (*1*). However, a cytosolic ACS could supply acetyl CoA for fatty acid elongation reactions and the biosynthesis of isoprenoids via the mevalonic acid (MVA) pathway. Evidence for the activity of the PDH bypass in plants under aerobic conditions has been obtained from pollen in which radiolabeled ethanol was converted into a variety of compounds including amino acids and lipids (*2*). In leaves, the potential for dedicating pyruvic acid to the PDH bypass was demonstrated from the purification of a high affinity PDC (*3*). Recently, direct evidence for the presence of PDH bypass for acetyl-CoA biosynthesis was obtained by feeding radiolabeled ethanol to *Arabidopsis* seedlings. Relative to wild type seedlings, ALDH mutants had greatly reduced incorporation rates of radiolabeled ethanol into fatty acids (*4*). However, direct measurements of the production of PDH bypass intermediates in leaves under normal aerobic conditions is lacking. In this study, we present new gas phase concentration and flux measurements of volatile intermediates derived from the PDH bypass from plants at the leaf, branch, and whole ecosystem scale.

Although acetone is emitted from all plant species studied to date and is one of the dominant VOCs released by many ecosystems (*5*), the metabolic origin of acetone in plants remains unclear (except in cyanogenic plants, which release both acetone and hydrogen cyanide in response to herbivory (*6*)). Attempts to demonstrate its production from acetoacetic acid, as has been shown in animals and bacteria, have generally failed (*7*). However, during $^{13}CO_2$ labeling experiments on needles of Gray pine (*Pinus sabiniana*), acetone was released with all three carbons showing up to 50% labeling (*8*), demonstrating that acetone is produced, in part, from recently photoassimilated carbon. In this study, we investigated the possibility that volatile isoprenoids and oxygenated VOCs including acetone are produced as a result of pyruvic acid metabolism. Because 1-C from pyruvate is typically lost during decarboxylation, while the other 2 C's are used for production of downstream metabolites, we used pyruvate-1-^{13}C and pyruvate-2-^{13}C labeling experiments to identify and examine the cellular fate of volatile pyruvate products.

We also report the development of an online technique for the quantification of pyruvic acid concentrations in air based on proton transfer reaction mass spectrometry (PTR-MS) and thermal desorption-gas chromatography−mass spectrometry (TD-GC-MS). We demonstrate the utility of these techniques by measuring pyruvic acid concentrations in the atmosphere of the tropical rainforest (TRF) biome at Biosphere 2.

Experimental Section

VOC concentration and flux measurements were made with a commercial high sensitivity PTR-MS instrument (IONICON Analytik, Innsbruck, Austria). The parent ions for each VOC studied were monitored with a 1 s dwell time and include m/z 45 (acetaldehyde), m/z 47 (ethanol), m/z 59 (acetone), m/z 61 (acetic acid), m/z 69 (isoprene), m/z 83 (C6 aldehydes and alcohols), m/z 89 (pyruvic acid), m/z 137 (monoterpenes), and m/z 205 (sesquiterpenes). For natural abundance and ^{13}C-labeling studies on individual leaves, the ions corre-

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10.1021/es903544p

Published on Web 03/08/2010

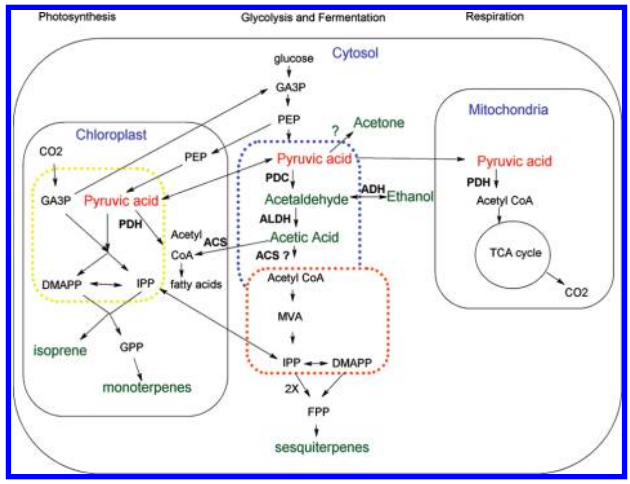

FIGURE 1. Simplified representation of VOC metabolism in photosynthetic plant cells. Abbreviations; PDC: pyruvate decarboxylase, PDH: pyruvate dehydrogenase, ALDH: aldehyde dehydrogenase, ACS: acetyl CoA synthetase, TCA cycle: tricarboxylic acid cycle, MVA: mevalonic acid, IPP: isopentenyl pyrophosphate, DMAPP: dimethylallyl pyrophosphate, FPP: farnesyl pyrophosphate, GA3P: glyceraldehyde 3-phosphate, PEP: phosphoenolpyruvic acid, MEP: 2-C-methyl-D-erythritol 4-phosphate. Yellow dotted circle: MEP pathway, blue dotted circle: PDH bypass, and red dotted circle: mevalonic acid pathway.

sponding to the VOC isotopologues with a single ^{13}C atom were also monitored. These were detected at m/z 46 (^{13}C-acetaldehyde), m/z 48 (^{13}C-ethanol), m/z 60 (^{13}C-acetone), m/z 62 (^{13}C-acetic acid), m/z 70 (^{13}C-isoprene), m/z 90 (^{13}C-pyruvic acid), m/z 138 (^{13}C-monoterpenes), and m/z 206 (^{13}C-sesquiterpenes). Thermal desorption-gas chromatography−mass spectrometry (TD-GC-MS) was used to verify the presence of pyruvic acid in TRF air. Technical details of the PTR-MS and TD-GC-MS configuration as well as the setup for ambient air concentration and branch flux measurements in the TRF can be found in the Supporting Information. Briefly, air from two heights (mid: 13 m and top: 26 m) within the TRF biome was pumped through heated (50 °C) Teflon tubing (1/4" O.D. 250' length) into the adjacent trace gas laboratory, where it was analyzed for VOC concentrations using PTR-MS and TD-GC-MS. Simultaneous PTR-MS measurements of VOC emissions from a single branch of a mango tree (*Mangifera indica*) inside the TRF were made using a 5 L Teflon branch enclosure during May 1−11, 2009. This branch was chosen because of its exposure to sunlight and proximity to an outside air port (external to Biosphere 2). Air was pumped into the enclosure after passing through a catalytic converter to destroy any hydrocarbons present. For single leaf gas exchange measurements, a custom-built glass enclosure (volume≈400 mL) with light and temperature control was used. A single mango tree leaf was removed, and the petiole was immediately recut under either distilled water (four leaves), 44.7 mM sodium pyruvate (four leaves), 44.7 mM sodium pyruvate-1-^{13}C (one leaf), or 44.7 mM sodium pyruvate-2-^{13}C (four leaves). Continuous measurements were acquired for at least 12 h. See the Supporting Information for additional experimental details.

Results

Whole Biome Concentration and Branch Flux Measurements. Whole biome VOC concentrations were similar at both mid and top heights (top height shown in the Supporting Information, Figure S1) and displayed a strong diurnal cycle with very high maxima occurring from midday to early afternoon. Maximum concentrations were approximately 120 ppbv (isoprene), 6 ppbv (monoterpenes), 6 ppbv (sesquiterpenes), 70 ppbv (acetone), 12 ppbv (acetaldehyde), 200 ppbv (ethanol), 7 ppbv (acetic acid), and 15 ppbv (pyruvic acid). Identification of pyruvic acid in TRF air was confirmed using TD-GC-MS by comparison with mass spectra in the National Institute of Standards and Technologies (NIST) database and by retention time and mass spectra comparison with a pure pyruvic acid standard (Supporting Information, Figures S2 and S3).

Despite using the TRF biome as a dynamic, flow through system, maximum VOC concentrations were approximately 5−30 times higher than observed in natural forest air. For example, in a tropical rainforest in Costa Rica, maximum noon-time concentrations were 6 ppbv (isoprene), 1 ppbv (monoterpenes), 2.5 ppbv (acetone), and 1.5 ppbv acetaldehyde (*5*). In a northern hardwood forest, average maximum sesquiterpene concentrations at noon were 0.02 ppbv (*9*). Concentrations measured inside the TRF biome at Biosphere 2 were substantially higher than in natural forest air due to (a) a long turnover time of the air and (b) a lack of UV light and gas phase oxidants, including ozone. To our knowledge, only two studies have attempted to measure pyruvic acid within a forest canopy. Using ion chromatography, maximum pyruvic acid concentrations of 0.06 and 0.266 ppbv were found in the Amazon (*10*) and Shenandoah National Park, Virginia (*11*), respectively. The maximum pyruvic acid concentrations of 15 ppbv and sesquiterpene concentrations of 6 ppbv measured in the TRF biome at Biosphere 2 demonstrate the usefulness of this facility for studying the dynamics of VOCs found in very low abundances in the natural atmosphere.

It is apparent that ambient concentrations of pyruvic acid, oxygenated VOCs, and volatile isoprenoids all share a similar diurnal pattern (Supporting Information, Figure S1), suggesting that they may be regulated by the same physiological processes and consistent with the possibility that pyruvate may serve as a common precursor. To verify the important role of vegetation in influencing atmospheric concentrations in the TRF system, branch emissions were measured continuously in parallel with ambient VOC concentration measurements. The results show significant emissions of all VOCs measured in the ambient air (Supporting Information, Figure S4). While emission rates of most of the compounds were consistent from day to day, emissions of monoterpenes and sesquiterpenes were more variable. However, for all compounds a strong diurnal pattern occurred with maximum emission rates during midday when sunlight and leaf temperature were at a maximum. Pyruvic acid and VOC emission rates displayed similar diurnal patterns, matching the observed diurnal patterns in whole biome concentrations.

Leaf Studies. Immediately after a leaf was placed in the enclosure, an initial burst in the emission rates of C6 aldehydes and alcohols as well as acetaldehyde, ethanol, acetic acid, and acetone occurred, likely as a wound response from the initial leaf cutting (data not shown) (*12*). When single leaves were placed in distilled water, emissions of isoprene generally followed assimilation/transpiration rates (Supporting Information, Figure S5), while acetaldehyde, acetic acid, monoterpene, and sesquiterpene emissions increased with extended illumination and remained elevated as assimilation decreased. When petioles of single detached leaves were placed in a 44.7 mM solution of sodium pyruvate, emissions of all VOCs increased relative to water-fed controls (Figure 2). Emissions of ^{13}C-labeled VOCs occurred at $^{13}C/^{12}C$ ratios consistent with natural abundance source. For example, at the peak in emission rates, the measured isotope ratios ($^{13}C/^{12}C$) for sesquiterpenes, monoterpenes, isoprene, and acetone were 15.4%, 10.6%, 5.7%, and 3.2%, respectively, which compare well with expected isotope ratios (16.6%, 11.1%, 5.5%, 3.3%; isotope distribution calculator, http://www.sisweb.com/mstools/isotope.htm). After several hours,

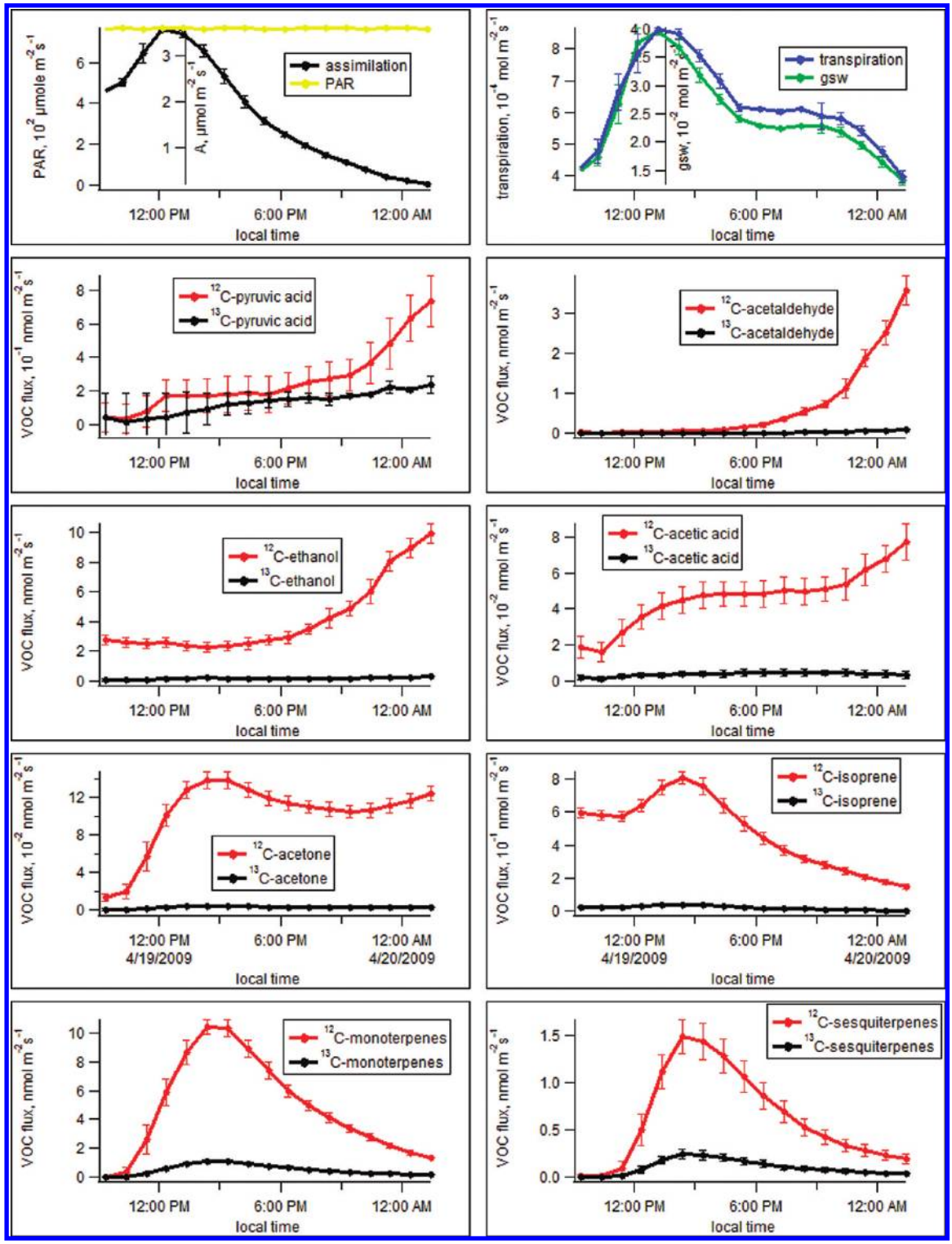

FIGURE 2. Emissions of VOCs and ^{13}C-labeled VOCs from a detached *Mangifera indica* leaf placed in 44.7 mM sodium pyruvate. Photosynthetically active radiation (PAR) and rates of net carbon assimilation, transpiration, and stomatal conductance to water (gsw) are also shown.

the maximum acetaldehyde, ethanol, acetic acid, acetone, monoterpene, and sesquiterpene emission rates were 3.6, 10.0, 0.1, 0.1, 10.4, and 1.5 nmol m^{-2} s^{-1}, respectively. These represent emission rates 2−120 times greater than those of leaves in distilled water (Figure 2 and Supporting Information, Figure S5). In repeated experiments with different leaves, considerable variability in the VOC emission rates was observed, but sodium pyruvate feeding always resulted in the enhancement of VOC emissions relative to distilled water controls. When data from the first hour were omitted (due to the initial emission burst), pyruvic acid emissions significantly correlated with acetaldehyde, acetic acid, and ethanol emissions, with linear correlation coefficients of 0.98, 0.95, and 0.97, respectively (Supporting Information, Figure S6). Correlations between pyruvic acid and acetone were weaker (correlation coefficient of 0.61) suggesting that there may be other important controls on acetone emissions.

Prior to the arrival of exogenous pyruvate-2-^{13}C,^{12}C-isoprene emissions from leaves in sodium pyruvate-2-^{13}C generally tracked assimilation rates. During the decline in

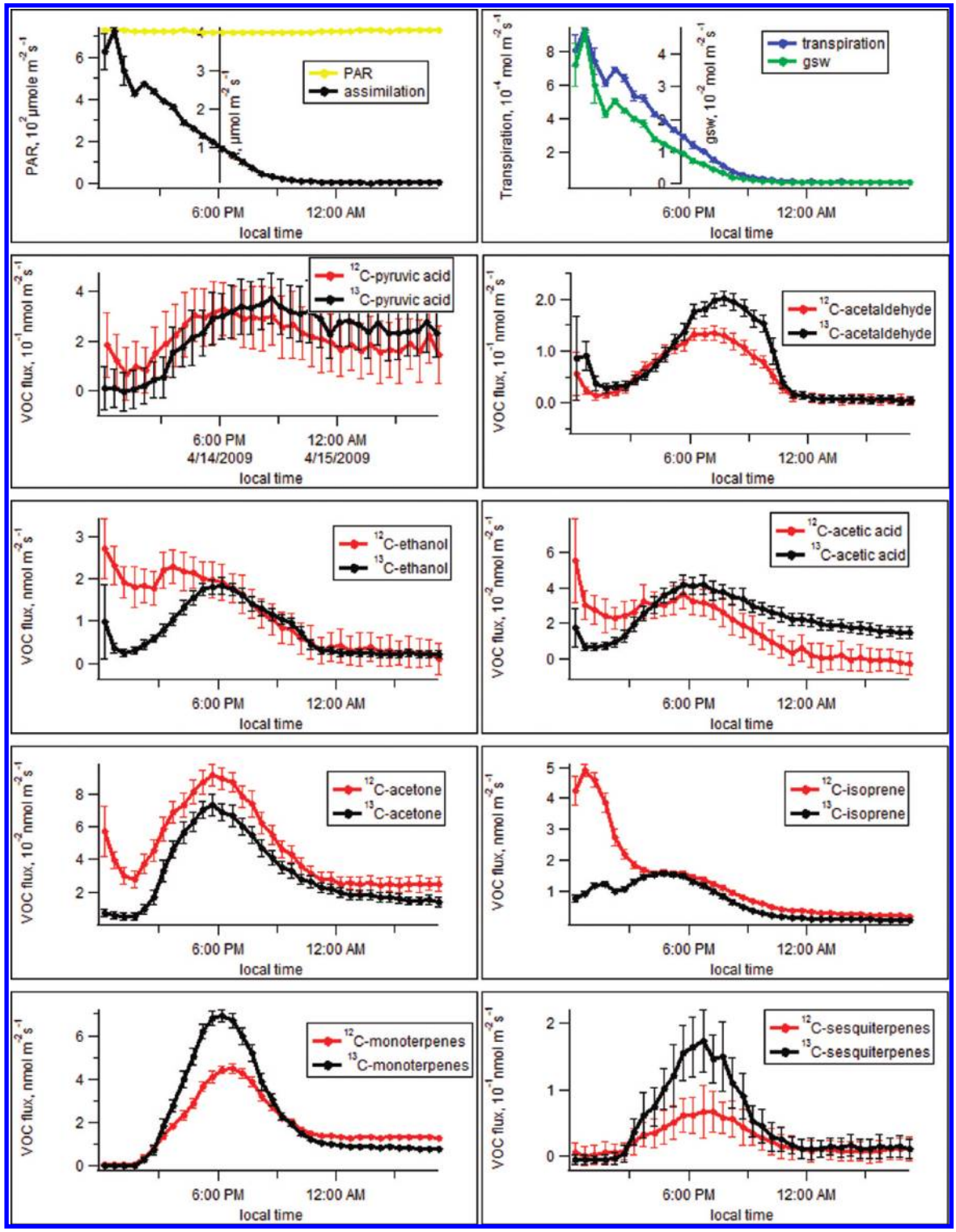

FIGURE 3. Emissions of VOCs and ^{13}C-labeled VOCs from a detached *Mangifera indica* leaf placed in 44.7 mM sodium pyruvate-2-^{13}C. Photosynthetically active radiation (PAR) and rates of net carbon assimilation, transpiration, and stomatal conductance to water (gsw) are also shown.

assimilation rate and ^{12}C-isoprene emissions, a pulse in ^{13}C-isoprene emissions occurred as the exogenous pyruvate-2-^{13}C arrived in the leaf. At this time, emission rates of all ^{13}C-labeled VOCs including ^{13}C-pyruvic acid were strongly enhanced, exceeding in most cases the ^{12}C-isotopologue emission rates (Figure 3). The measured stable carbon isotope ratios ($^{13}C/^{12}C$) were strongly enriched in ^{13}C derived from the sodium pyruvate-2-^{13}C. At the peak in ^{13}C-VOC emission rates, isotope ratios ($^{13}C/^{12}C$) for sesquiterpenes, monoterpenes, isoprene, acetone, acetaldehyde, acetic acid, and ethanol were 266%, 157%, 100%, 78%, 154%, 124%, and 100%, respectively.

In the single experiment with 44.7 mM sodium pyruvate-1-^{13}C, small emissions of ^{13}C-labeled pyruvic acid from the leaf were observed after several hours (Figure 4). Significant emissions of ^{13}C-labeled isoprenoids occurred, resulting in increased stable carbon isotope ratios relative to natural abundance sources: 28.8%, 59.5%, and 32.9% compared to 5.5%, 11.1%, 16.6% for isoprene, monoterpenes, and sesquiterpenes, respectively. However, significant emissions of

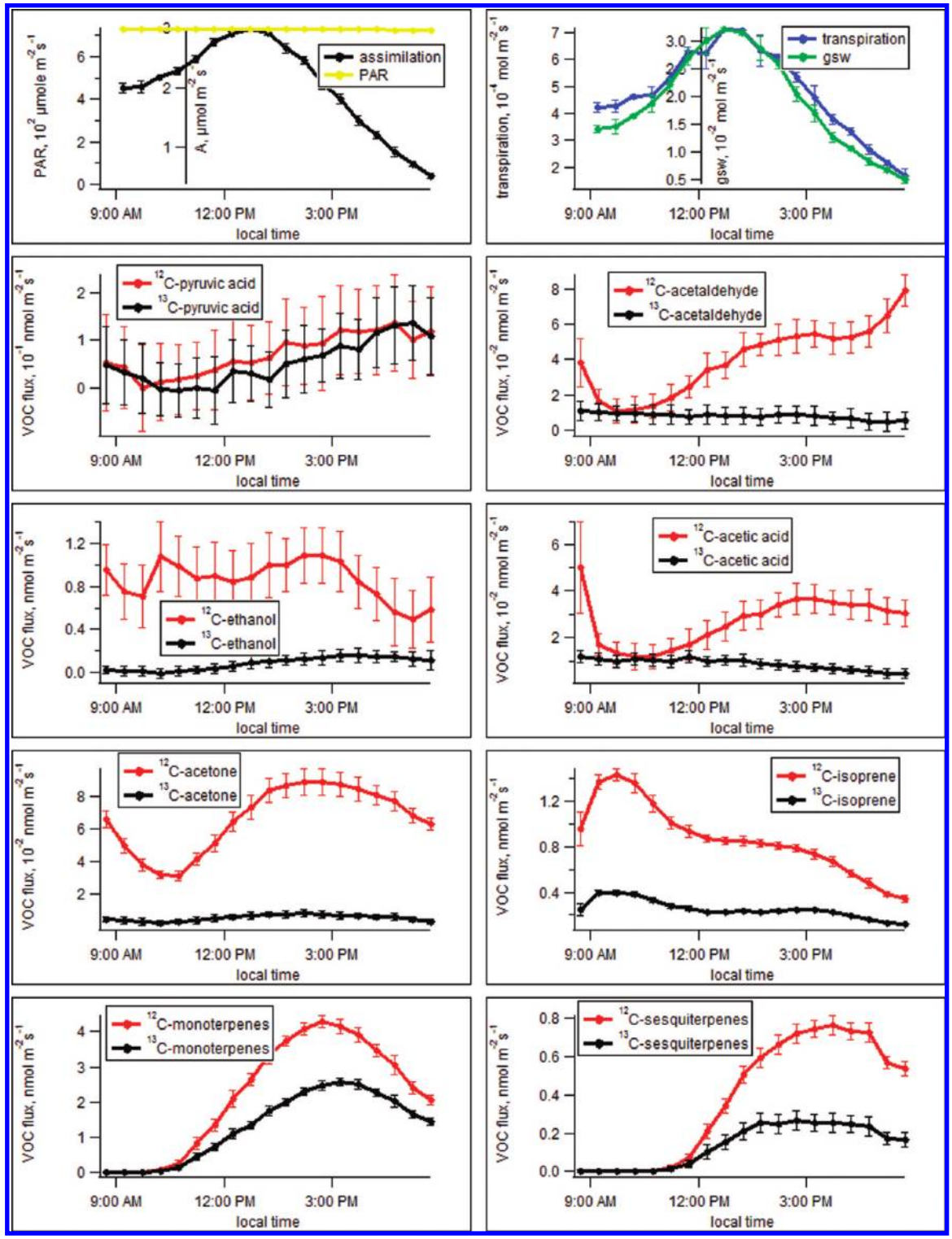

FIGURE 4. Emissions of VOCs and ^{13}C-labeled VOCs from a detached *Mangifera indica* leaf placed in 44.7 mM sodium pyruvate-1-^{13}C. Photosynthetically active radiation (PAR) and rates of net carbon assimilation, transpiration, and stomatal conductance to water (gsw) are also shown.

^{13}C-labeled acetaldehyde, ethanol, acetic acid, and acetone emissions were not observed.

Discussion

In this study, the fact that pyruvic acid is a precursor in the biosynthesis of volatile isoprenoids and several oxygenated VOCs is demonstrated by the increase in emissions upon pyruvate feeding and, more importantly, the appearance of ^{13}C-label in these VOCs from the 2-C of pyruvate (Figure 3). Oxygenated VOCs may be produced from pyruvic acid by the PDH bypass in the cytosol, while pyruvic acid and isopentenyl pyrophosphate (IPP) can be transported into the chloroplast and feed directly into the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprene and monoterpene production (Figure 1). The bias in ^{13}C-monoterpene production over ^{13}C-isoprene may reflect the fact that terpene synthases have a greater affinity for dimethylallyl pyrophosphate (DMAPP) than does isoprene synthase (*13*, *14*).

Acetyl CoA for sesquiterpene biosynthesis in the cytosol may derive from the exchange of intermediates such as IPP between the cytosol and chloroplasts or possibly from acetate produced by the PDH bypass.

The lack of ^{13}C label in oxygenated VOCs following sodium pyruvate-1-^{13}C leaf feeding suggests the involvement of the PDH bypass; the carbon lost as CO_2 in the PDC reaction is that in the 1-C position of pyruvate. The absence of label in acetone suggests that acetone production in plants, like that of acetaldehyde, acetic acid, and ethanol, is associated with the decarboxylation of pyruvate. Although the mechanism for acetone production from pyruvate in leaves needs to be explored in more detail, one possible mechanism is the decarboxylation of the 1-C of pyruvate accompanied by a methyl transferase reaction. Surprisingly, emissions of ^{13}C-labeled isoprene, monoterpenes, and sesquiterpenes occurred at a much higher rate than expected for natural abundance carbon sources (Figure 4). One possible explanation for this is that a portion of the ^{13}C-label released from pyruvate-1^{13}C as $^{13}CO_2$ is reassimilated by the Calvin cycle and subsequently incorporated into isoprene and monoterpenes via the plastidic MEP pathway. Because sesquiterpenes derive from the MVA pathway in the cytosol, incorporation of label into sesquiterpenes may involve exchange of labeled IPP between cellular compartments. Therefore, our observations support previous studies demonstrating that volatile isoprenoids are derived from two pathways in plants, the MVA pathway in the cytosol and the plastidic MEP pathway, and linked by the exchange of common intermediates such as IPP and pyruvic acid (*15*).

Although the affinity of PDH for pyruvic acid greatly exceeds that of PDC (*16*), should pyruvic acid concentrations become elevated and/or mitochondrial PDH become less active in leaves, the PDC reaction may be able to compete with the PDH reaction. In the light, carbon fixation in chloroplasts provides triose-phosphate to the cytosol, including glyceraldehyde-3-phosphate which can then be converted to pyruvic acid by glycolysis. It is generally accepted that day respiration (mitochondrial nonphotorespiratory CO_2 production) in C3 leaves is suppressed relative to respiration in the dark (*17*). This is partially attributed to the light induced inhibition of mitochondrial PDH (*18*). Although the plastidial PDH may be more active in the light than in the dark (leading to increased fatty acid biosynthesis), photosynthesis and reduced day respiration may lead to the accumulation of primary photosynthetic products like pyruvic acid (*19*). As pyruvic acid accumulates in the light, one consequence could be increased production rates of volatile isoprenoids and oxygenated VOCs. This agrees with many observations that the emissions of these compounds from plants are light and temperature dependent, a fact that has enabled the development of global biogenic VOC emission models (*20*). While light dependent emissions of both isoprene and monoterpene have been clearly demonstrated (*21*), the light dependence of sesquiterpene (*22*) and oxygenated VOC (*23*) emissions remains uncertain. Although other important factors, including possible diurnal patterns in enzyme activities involved in their production (*24*), control the production of isoprenoids, our results suggest that the biosynthesis of volatile isoprenoids and some oxygenated VOCs (acetaldehyde, acetone, acetic acid, and ethanol) may demonstrate a light dependency at least in part due to the accumulation of pyruvic acid during photosynthesis. However, because there are other sources of pyruvic acid, including glycolysis, that are not light dependent, a complete dependence of the emission rates on light is not expected. This model is supported by previous studies that demonstrated a diurnal variation of pyruvic acid concentrations in leaves. For example, pyruvic acid concentrations in poplar leaves peak during midday (*25*). Also, with extended illumination of leaves, the concentration of pyruvic acid continues to increase (*26*).

Natural emissions of VOCs from the terrestrial biosphere are estimated to be an order of magnitude larger than anthropogenic emissions globally with volatile isoprenoids and oxygenated VOCs as the dominant species (*20*). The results from this study may aid in the development of mechanistic predictive models for the emissions of VOCs from the biosphere to the atmosphere for use in air quality and climate models.

Acknowledgments

This research was supported by a joint postdoctoral fellowship program between Biosphere 2 Earth Science division and the National Center for Atmospheric Research. We would like to kindly thank John Adams and Joey Martinez at Biosphere 2 for providing much of the required infrastructure needed in this study. Instrumentation support is acknowledged from NSF (CHE 0216226) and from Edward P. Bass.

Supporting Information Available

More information on the technical details of the PTR-MS and TD-GC-MS configuration, the setup for ambient air concentration and branch flux measurements in the TRF, leaf feeding experiments, and additional figures including Figure S1: diurnal concentration patterns of VOCs near the top of the TRF biome (26 m), Figure S2: bar plot of the dominant ions produced during electron-impact ionization mass spectra of pyruvic acid, Figure S3: pyruvic acid sample peaks measured with TD-GC-MS, Figure S4: diurnal patterns of branch level emission rates of VOCs from a *Mangifera indica* tree in the TRF biome, Figure S5: emissions of VOCs and ^{13}C-labeled VOCs from a detached *Mangifera indica* leaf placed in distilled water, and Figure S6: linear correlation between VOC and pyruvic acid emissions during sodium pyruvate leaf feeding. This material is available free of charge via the Internet at http://pubs.acs.org.

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ES903544P